Decoding therapeutic roles of adipose tissue-derived stromal cells and their extracellular vesicles in liver disease

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APPENDIX A

SUMMARY

Mesenchymal stromal cells (MSC) are therapeutic cells that, upon the systemic infusion, have the ability to home to the injured liver. MSC improve liver function and ameliorate fibrosis, rendering a promising strategy to treat patients with end-stage liver disease. Although MSC are capable to differentiate into hepatocytes *in vitro*, the MSC-induced therapeutic effects on acute and chronic liver disease appear based on paracrine factors they produce, mediated via the release of trophic factors and extracellular vesicles (EVs). Various trophic molecules have been studied as carriers of the MSC therapeutic effects, while such function for MSC-derived extracellular vesicles has not been yet thoroughly investigated. EVs carry a variety of regulatory nucleic acids and proteins and are able to deliver this cargo to target cells. Cargo of EVs may modulate key cellular processes, such as transcription, post-transcriptional modification and signal transduction in the recipient cells. In this thesis, we established the therapeutic potential of EVs from human adipose tissue-derived stromal cells (hASC-derived EVs) in murine, cellular and tissue (*ex vivo*) models of acute and chronic liver disease.

Chronic liver disease is commonly accompanied by the development of liver fibrosis and hepatic stellate cells (HSC) are considered to be the main drivers of this process that impairs liver function. HSC are located in the space of Disse and are referred as quiescent HSC in the healthy liver. As a result of chronic liver injury, HSC become activated, start to proliferate and produce excessive amounts of extracellular matrix (ECM) proteins, as well as cytokines, chemokines and mitogenic growth factors. Experimental liver fibrosis in rodents is alleviated after systemic administration of MSC yet the type and cellular origin of chemoattractants that cause homing of MSC to the liver are largely unknown. In **Chapter 2**, we found that activated HSC attract hASC to the liver through the secretion of specific chemokines. hASC migrated only to activated HSC and not to quiescent HSC and neither to hepatocytes, highlighting that activation of HSC is a key factor in attraction of hASC to the fibrotic liver. Selective inhibition of chemokine receptors by pharmacological compounds revealed that CXCR2 and CXCR3 are both involved in MSC migration to activated HSC.

hASC are able to suppress liver fibrogenesis, but the underlying mechanisms are largely unknown. In **Chapter 3,** we show that hASC secrete factors (the "hASC-secretome") that effectively suppress HSC activation *in vitro* and liver fibrosis *in vivo*. These anti-fibrotic properties appear almost exclusively in the EV-fraction, and are not found in the EV-free fraction that contains the trophic factors. These

data indicate that hASC-derived EVs suppress hepatic fibrosis *in vitro*, *in vivo* and *ex vivo*. EVs carry a great variety of different proteins and various types of RNA, such as miRNAs. Our primary miRNA analysis revealed that hASC-derived EVs contain over 1,000 unique types of miRNAs. Pathway enrichment analysis indicated various associations between these miRNAs and pathways regulating fibroblast function and fibrosis, such as MAP Kinase and FoxO signaling pathways.

Acute liver failure is a life-threatining disease where the damaged liver fails within days to weeks. Acute liver damage is associated with inflammation and massive hepatocyte loss. In **Chapter 4**, we challenged the potential of hASC-derived EVs to attenuate acute liver damage in acetaminophen (APAP)- and carbon tetrachloride (CCl_4)-induced murine models of acute liver injury. Both the prophylactic, as well as the therapeutic application of hASC-derived EVs rescued the acute liver injury and suppressed the inflammation in these murine liver disease models. Moreover, hASC-derived EVs suppressed the early onset of liver fibrosis induced by the single high-dose of CCl_4, in line with observations made in Chapter 3.

Hepatic steatosis is the accumulation of fat in the liver. As a consequence of a Western diet, the physiological equilibrium in hepatocyte lipid metabolism is disturbed and uptake and/or *de novo* synthesis of fatty acids is increased relative to fatty acid oxidation. This stimulates the synthesis of triglycerides in the liver to dispose the excess free fatty acids resulting in steatosis. In **Chapter 5**, we show that hASC-derived EVs reverse hepatic steatosis in mice placed on a Western diet for 6 weeks. The 6-week Western diet induced triglyceride accumulation in the liver, but did not cause liver damage, inflammation and fibrosis yet. Weekly administration of hASC-derived EVs in the last 3 weeks of the Western diet feeding lowered hepatic lipid accumulation. Thus, this mild steatosis model of NAFLD showed that hASC-derived EVs directly suppress hepatic steatosis, independent of the co-existence of inflammation and/or fibrosis.

In conclusion, CXCR2 and CXCR3 are prospective targets for the improvement of hASC homing in liver disease. hASC-derived EVs are anti-fibrotic and anti-steatotic, and are able to suppress acute hepatocyte injury. However, mechanisms of these beneficial effects remain to be established in detail in future studies. This thesis provides a platform for the future application of hASC-derived EVs in the treatment of acute and chronic liver disease.

NEDERLANDSE SAMENVATTING

Mesenchymale stromale cellen (MSC) zijn therapeutische cellen die na systemische infusie het vermogen hebben om naar de zieke lever te gaan. MSC verbeteren de leverfunctie en herstellen fibrose, wat een veelbelovende strategie is voor de behandeling van patiënten met vergevorderde leverziekte. Hoewel MSC onder gecontroleerde omstandigheden *in vitro* in levercellen (hepatocyten) kunnen differentiëren, lijken de therapeutische effecten van MSC in acute en chronische leverziekte vooral afkomstig te zijn van paracriene factoren die ze produceren. Effecten van MSC worden gemedieerd via de afgifte van kleine eiwitten (trofische factoren) en extracellulaire vesikels (EV's). De therapeutische effecten van MSC zijn al toegeschreven aan verschillende trofische factoren, maar dergelijke functies zijn nog niet grondig onderzocht voor MSC-geproduceerde EV's. EV's bevatten een verscheidenheid aan regulerende nucleïnezuren en eiwitten en zijn in staat om deze aan zieke organen af te leveren. De bestanddelen van EV's kunnen belangrijke cellulaire processen moduleren, zoals transcriptie, post-transcriptionele modificatie en signaaltransductie in de ontvangende cellen. In dit proefschrift hebben we het therapeutische potentieel van EV's van MSC onderzocht, in het bijzonder van menselijk vetweefsel afkomstige stromale cellen (hASC-geproduceerde-EV's) in muis-, cel- en weefsel- (*ex vivo*) modellen van acute en chronische leverziekte.

Chronische leverziekte gaat vaak gepaard met de ontwikkeling van leverfibrose. Hepatische stellaatcellen (HSC) worden beschouwd als de belangrijkste oorzaak van dit proces dat de leverfunctie schaadt. HSC bevinden zich in de ruimte van Disse en worden in de gezonde lever aangeduid als "rustende" HSC. Als gevolg van chronisch leverletsel worden HSC geactiveerd en gaan deze cellen prolifereren en produceren ze buitensporige hoeveelheden extracellulaire matrix (ECM) eiwitten, evenals cytokinen, chemokinen en groeifactoren. Systemische toediening van MSC onderdrukt leverfibrose bij knaagdieren, maar het type en de cellulaire oorsprong van chemoattractanten die MSC naar de lever brengen, zijn grotendeels onbekend. In **Hoofdstuk 2** ontdekten we dat geactiveerde HSC specifieke chemokinen uitscheiden die ervoor zorgen dat hASC naar de zieke lever trekken. hASC migreerden alleen naar geactiveerde HSC en niet naar rustende HSC en ook niet naar hepatocyten, wat aangeeft dat activering van HSC een belangrijk proces is voor het aantrekken van hASC naar de fibrotische lever. Selectieve remming van chemokinereceptoren door farmacologische stoffen onthulde dat de receptoren CXCR2 en CXCR3 betrokken zijn bij MSC-migratie naar geactiveerde HSC.

hASC kunnen leverfibrose onderdrukken, maar de onderliggende mechanismen zijn grotendeels onbekend. In **Hoofdstuk 3** laten we zien dat hASC factoren uitscheiden (het 'hASC-secretoom') die HSC-activering *in vitro* en leverfibrose *in vivo* effectief remmen. Deze anti-fibrotische eigenschappen zitten bijna uitsluitend in de EV-fractie en worden niet gevonden in de EV-vrije fractie die de trofische factoren bevat. Deze gegevens geven aan dat hASC- geproduceerde-EV's lever fibrose *in vitro*, *in vivo* en *ex vivo* onderdrukken. EV's bevatten een grote verscheidenheid aan verschillende eiwitten en verschillende soorten RNA, zoals miRNA's. Onze miRNA-analyse onthulde dat hASC- geproduceerde-EV's meer dan 1.000 verschillende soorten miRNA's bevatten. Onze signaleringsroute analyse (pathway enrichment analysis) wees op verschillende associaties tussen deze miRNA's en routes die de fibroblastfunctie en fibrose reguleren, zoals MAP Kinase- en FoxO-signaleringsroutes.

Acuut leverfalen is een levensbedreigende ziekte waarbij de beschadigde lever binnen enkele dagen tot weken faalt. Acute leverschade wordt geassocieerd met ontsteking en massaal verlies van hepatocyten. In **Hoofdstuk 4** hebben we getest of hASC-geproduceerde-EV's ook in staat zijn acute leverschade door paracetamol (APAP) of tetrachloormethaan (CCl_4) in muizen kan tegengaan. Zowel de profylactische als de therapeutische toepassing van hASC-geproduceerde-EV's beschermden de lever tegen de acute leverbeschadiging en onderdrukten de ontsteking in deze muismodellen van leverziekte. Bovendien onderdrukten hASC-geproduceerde--EV's de eerste kenmerken van leverfibrose geïnduceerd door CCl_4, in overeenstemming met de resultaten beschreven in hoofdstuk 3.

Leververvetting (steatose) is de ophoping van vet in de lever. Als gevolg van een westers dieet wordt het fysiologische evenwicht in het vetmetabolisme van de hepatocyten verstoord en neemt de opname en/of *de novo* synthese van vetzuren toe ten opzichte van de vetzuuroxidatie. Dit stimuleert de vorming van triglyceriden in de lever om de overtollige vrije vetzuren af te voeren, wat uiteindelijk resulteert in steatose. In **Hoofdstuk 5** laten we zien dat hASC-geproduceerde-EV's leververvetting tegengaan bij muizen die gedurende 6 weken een westers dieet te eten kregen. Het westerse dieet van 6 weken induceerde ophoping van triglyceriden in de lever, maar veroorzaakte nog geen leverschade, ontsteking of fibrose. Wekelijkse toediening van hASC-geproduceerde-EV's in de laatste 3 weken van het westerse dieet verminderde ophoping van lipiden in de lever. Aldus toonde dit milde steatosemodel van niet-alcoholische vette leverziekte

(NAFLD) aan dat hASC-geproduceerde--EV's leversteatose direct onderdrukken, onafhankelijk van de aanwezigheid van ontsteking en/of fibrose.

We kunnen nu dus concluderen dat CXCR2 en CXCR3 factoren zijn die hASC-migratie naar de lever bij leverziekte kunnen stimuleren. Verder zijn hASC-geproduceerde-EV's anti-fibrotisch en anti-steatotisch en kunnen acuut leverletsel onderdrukken. De mechanismen van deze gunstige effecten moeten echter in toekomstige studies verder in detail worden bepaald. Dit proefschrift biedt een platform voor de toekomstige toepassing van hASC-geproduceerde-EV's bij de behandeling van acute en chronische leverziekte.

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LIST OF PUBLICATIONS

Afsharzadeh D, Sydor S, Gore E, Friedrich J, Krenning G, Van Rijn P, Olinga P, Canbay A, Bechmann LP, Harmsen MC and Faber KN. Adipose tissue-derived stromal cells suppress liver fibrosis in an extracellular vesicle-mediated fashion. *Submitted*

Afsharzadeh D, Sydor S, Saeed A, Canbay A, Olinga P, Bechmann LP, Harmsen MC and Faber KN. Adipose tissue-derived stromal cells are attracted to activated hepatic stellate cells through CXCR2- and CXCR3-mediated signalling. *Submitted*

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